Report of Drs. Cole and Avery (assisted by Drs. Abernethy, Dubos, Goebel, Goodner, Hotchkiss, Horsfall, C. MacLeod, and Stillman)

Therapy of pneumonia. Shortly before the opening of this hospital, Dr. Osler said, "Pneumonia is a self-limited disease, which can neither be aborted nor cut short by any means at our command." Attempts to develop methods for successfully treating this disease have engaged the attention of various members of the staff ever since the hospital was opened. This idea has been in the background of a large amount of experimental work which has yielded important results of general significance, at first sight not related directly to the larger problem, but nevertheless possibly having much importance for its ultimate solution. Such, for instance, have been the demonstration of the important part which carbohydrates play in immunological phenomena, and more recently the demonstration of the role of lipoids in immunity reactions. Indeed, such apparently unrelated matters as increase of knowledge concerning gas exchange in the blood and new knowledge concerning the chemistry of sugars have had the same origin, and many other similar examples might be mentioned.

The main problem, however, has not been lost sight of, and many procedures which have been proposed for the treatment of pneumonia have been investigated--not only those proposed here, but those which have been introduced elsewhere. Some of the latter have been studied, not because they seemed of great value, but because they were being widely employed and because the resources of our hospital permitted a kind of intensive investigation not possible elsewhere. These efforts grew out of a realization of the more immediate obligation of the hospital to the medical profession and the public. Such studies were those concerning certain chemotherapeutic

drugs such as optochin, of oxygen therapy, of diathermy, and more recently of artificial pneumothorax. Conclusive evidence was brought to discredit certain of these measures, such as diathermy, while in the case of others their exact value was sharply defined.

During the past two years an investigation of the method of artificial pneumothorax in the treatment of pneumonia has been made, and a report on the first cases treated has been made by Abernethy, Horsfall, and MacLeod. The results of this study indicate that the injection of small amounts of air into the pleura may be effective in certain cases in relieving the severe pain, and so may indirectly be of value, but that the procedure has no apparent effect on the course of the disease, and indeed, complete collapse of the lung, which has been advocated, may give rise to complications which are distinctly harmful.

The first therapeutic measure which was studied in the hospital was treatment with immune horse serum, and this study has been continued up to the present time. It was undertaken because experimental studies in animals indicated strongly that under certain conditions, at least, it might be of value, and the first practical application made in this hospital indicated what these conditions might be. The work has been continued all these years in order that those conditions might be accurately defined, that the method might be developed so as to have a wider application, and finally, that evidence might be obtained which would be sufficiently convincing to influence its general adoption. The work was continued in the face of some discouragement, but the main facts have now been established, and the great value of Type I immune serum in the treatment of cases due to the homologous organism has now received general recognition. Four hundred and sixty-two cases of Type I pneumonia have now been treated in this hospital, with

a mortality rate of 10.5 per cent. Of the cases treated within the first three days, the mortality rate has been 4.8 per cent, in those treated on the fourth day or earlier, 8.2 per cent, on the fifth day or earlier, 8.6 per cent, and in those treated after the fifth day, 19.5 per cent. Of the last 25 patients, all treated with concentrated serum and most of them treated early, not one has died. When it is remembered that the general mortality rate of Type I pneumonia not treated with serum has been not less than 20 per cent, more likely 25 per cent, the effectiveness of serum treatment in this condition is obvious.

Largely through the influence of this hospital, Type I pneumonia is coming to be recognized as a specific infectious disease. Of this condition not less than 25,000 persons now die each year in this country, more than twice as many as ever died of typhoid fever when it was most prevalent. The first serum used was made by the hospital, but later the preparation of the serum was undertaken by the New York State Board of Health, and through the cooperation of Dr. Wadsworth, Director of the Laboratory of the State Board of Health, we have received free of charge all the serum we have used. This serum has cost the State many thousands of dollars.

During the past few years a campaign has been carried out by the State Board of Health of Massachusetts to promote the use of this serum and to assist the doctors in administering it. A similar campaign is now being undertaken by the New York State Board of Health, and the serum is being used extensively in England. From the very beginning, the Hospital has not engaged in efforts rapidly to extend its use, feeling that our proper function should be to bring evidence by demonstration of its usefulness, to make every effort to have it used in a proper and scientific manner, and especially to learn more about its mode of action and to devise

methods for adapting this or other specific methods to the treatment of other types of pneumonia.

The immunological significance of the lipids of antipneumococcus sera. (Goodner and Horsfall) Antipneumococcus horse and rabbit sera exhibit many contrasting properties. The more outstanding of these are shown in the following table:

	Antipneumococcus Horse Serum	Antipneumococcus Rabbit Serum
Precipitate with capsular polysaccharide 1:10,000		
Macroscopic	Granular cake	Translucent disc
Microscopic	Granular	Hyaline
Complement fixation with capsular polysaccharide	−	+
Passive sensitization of guinea pig to capsular polysaccharide	−	+
Capsular "Quellung" or swelling phenomenon	±	+
Protective prozone	+	−
Precipitation of immune fraction by 9 volumes of distilled water	+	−
Reaction with degradation products of capsular polysaccharide	+	−

There are, in addition, two further striking species dissimilarities. First, the capsular polysaccharide, of itself, is antigenic in the horse but not in the rabbit. The second point is the relation of the reactivity of these sera to lipids, the subject of this report.

1. Antipneumococcus sera lose their properties of specific agglutination and precipitation after extraction with lipid solvents.

2. Providing due precautions have been taken against denaturation of the proteins, this treatment in no way alters the capacity of the antibody to combine with the antigen. This is evidenced by specific absorption with pneumococci and by the capacity of extracted antipneumococcus rabbit serum to give complement fixation with the capsular polysaccharides. Neither of these phenomena are dependent upon flocculation. Moreover, if pneumococci are first treated with lipid-extracted serum, subsequent addition of whole serum fails to bring about agglutination.

3. The agglutinating and precipitating properties of immune sera may, however, be restored in vitro by the addition of certain lipids. Thus antipneumococcus horse serum regains its flocculating properties on the addition of very small amounts of lecithin, while those of antipneumococcus rabbit serum return upon the addition of cephalin. The quantity of phosphatide which is essential for this restoration is of the order of 0.025 mg. per cc. of serum, an amount too small for accurate determination by present methods.

4. If to the extracted serum cholesterol is first added, the further addition of lecithin or cephalin respectively does not restore the secondary properties.

5. Antipneumococcus sera from ten species of animals have been investigated in these respects. Those from man, the mouse, the goat, the cat and the dog are analogous to that of the horse; that is, lecithin is required for restoration of secondary flocculating properties after lipid extraction. None give complement fixation with pneumococci or the capsular polysaccharides. In these animals the capsular polysaccharides are antigenic. On the other hand, antipneumococcus sera from guinea pigs, rats, and sheep possess the attributes of that from the rabbit. Secondary

properties are restored by the addition of cephalin. Complement fixation is obtained. In none of these animals are the capsular polysaccharides antigenic in their free form. No antipneumococcus serum has been found which does not fall into one of these two groups.

6. Chemical analyses carried out upon precipitates obtained from the interaction of antipneumococcus sera and the specific polysaccharides show that from 5 to 80 per cent of the total mass of the precipitate may consist of lipid. These large amounts of lipid are readily determined by analytical methods. It is believed that they are adsorbed on the immune aggregates.

7. In the equivalence zone, that is, the range of combinations of antigen and antibody in which both are present in dissociated form, the entrained lipids in the immune precipitate consist of cholesterol, cholesterol esters, and phosphatides. No neutral fat has been found. The capacities of these lipids to be adsorbed is in the order given.

8. There is no characteristic quantitative difference in the lipid pattern either of the original horse and rabbit sera or of the immune precipitates derived therefrom. There is, however, a qualitative distinction in the adsorbed phosphatides. With immune horse serum cephalin is found in the precipitates, while with the immune rabbit precipitates the phosphatide is lecithin. The adsorbed phosphatides appear to occupy a position paradoxical to those of the essential. Thus with immune horse serum traces of lecithin are required for precipitation, but the mass of phosphatide in the immune precipitate is cephalin. Conversely, with immune rabbit serum minute amounts of cephalin are essential for restoration of activity but large amounts of lecithin are found in the immune precipitates.

9. The addition of various lipids tends to modify the protective action of antipneumococcus sera, although here again there are marked

species differences. Thus neither cholesterol, cephalin nor lecithin affects the protective properties of antipneumococcus rabbit serum. On the other hand, the protection expected with antipneumococcus horse serum is blocked by both cholesterol and cephalin. It may be noted that both of the latter are "fixed" in the immune horse aggregates. Since cephalin is not fixed by rabbit aggregates, no action would be expected. The failure of cholesterol to inhibit the protection with immune rabbit serum is explained by the fact that lecithin and cholesterol are antagonistic.

The evidence thus far obtained and briefly summarized in the foregoing paragraphs permits the elaboration of the following conception.

a. In these immune reactions, certain lipids are <u>essential</u> to the secondary phenomena of flocculation (precipitation and agglutination) but not concerned with the primary and fundamental specific union of antigen and antibody. For antipneumococcus sera these lipids are phosphatides and have no specificity in the usual immunological sense, but are rather determined by the species source of the immune serum. In nature the antibody protein probably exists in combination with these lipids, and many of the natural properties of the antibody, as, for example, its solubility, are largely conditioned by the lipid portion of the molecule.

b. Certain other lipids are <u>bound</u> or fixed by the immune aggregate, but are <u>non-essential</u> for the visible phenomena of precipitation and agglutination. In one early phase in the course of flocculation, the immune aggregates behave as colloidal particles, and as such possess the property of adsorptive gels. There may or may not be a large variety of substances adsorbed on these particles, but it is certain that the lipids are taken down rather readily, and, as has been determined, constitute a significant portion of the final mass of the aggregate. In this process

the amount of lipid adsorbed bears, in general, a definite relationship to the properties of the milieu, and thus follows the general laws of adsorptive phenomena. In addition there is a conspicuous lack of selectivity except in two respects: first, certain lipids are more readily adsorbed than others; second, the non-essential phosphatides are again characteristic of the species, but are the opposites of the essential phosphatides. Since the amounts of these bound but non-essential lipids are not inconsiderable, they contribute in a striking sense to the properties of the immune aggregates. Moreover, they play a decisive role in the protective action of the immune sera.

The effect of enzymes on the antigenicity of pneumococcus vaccines (Dubos). Under appropriate conditions, the immunization of rabbits with "vaccines" prepared from cultures of virulent pneumococci may bring about the production of an antibody--a precipitin--directed against the type-specific capsular polysaccharide of the strain used in the vaccine. It has been shown, however, that this polysaccharide itself does not function as an antigen in the rabbit--at least not in the form in which it has been chemically extracted from the bacterial cells. All attempts to obtain the hypothetical "capsular polysaccharide antigen" in the form of a solution have so far failed, and this failure has rendered impossible a study of the chemical structure of the antigen in question. It was thought that the problem could be attacked in an indirect way by the use of known enzymes which would inactivate the type-specific antigen and reveal thereby the existence and nature of certain groups and linkages characteristic of its constitution.

Heat-killed or formalized suspensions of virulent pneumococci were used as source of antigens; the materials were tested for antigenic

activity by the intravenous injection of rabbits, and the presence of the antibody recognized by the occurrence of a precipitin reaction between the immune serum and high dilutions of the type-specific capsular polysaccharides.

Gram-positive cocci are known to be resistant to the action of common enzymes and we could confirm the fact that following the action of crystalline trypsin and chymotrypsin, and of commercial preparations of pepsin, papain, pancreatic lipase and phosphatase, the cells in a pneumococcus vaccine remained gram-positive, and, in the cases where it was tested, retained their specific antigenicity.

Two sets of observations served as leads for the discovery of enzymes capable of rendering ineffective in rabbits the "capsular polysaccharide antigen": (1) Previous studies from this department have established that rabbits injected intradermally fail to produce type-specific precipitins, thus differing from those immunized intravenously. This suggested as a possible explanation the existence in the skin of some principle capable of inactivating a part, at least, of the pneumococcus antigen. (2) It has also been observed that rabbits immunized intravenously with autolysates--or bile solutions--of pneumococci failed to respond with the usual formation of type-specific precipitins. This again suggested that the processes of autolysis and bile solution resulted in an inactivation of the antigen.

1. As stated above, rabbits immunized by repeated intradermal injections fail to respond with the formation of the capsular polysaccharide precipitin. To study this matter further, heat-killed cells of virulent pneumococci were injected at different sites into the skin of a rabbit. These injected areas were excised at different intervals of time and

microscopic preparations of tissue fragments were stained by the Gram technique. As early as 24 hours after injection, many pneumococci were found to have undergone a process of extracellular digestion, some of them becoming much smaller, but remaining gram-positive, others, on the contrary, losing the ability to retain the Gram stain, but maintaining their morphology. Many of the gram-negative cells had been engulfed by the leucocytes. This process progressively increased during the second and third days, until, on the fifth day, only gram-negative bacterial debris could be detected. These observations indicated the secretion by leucocytes of a ferment capable of lysing heat-killed pneumococci.

An attempt was made to isolate this principle, and study its properties. The following technique gave very dependable, and relatively pure preparations. An exudate consisting mostly of P.M.N. cells was obtained by injecting aleuronate into the pleural cavity of a rabbit. The cells were centrifuged, resuspended in saline and allowed to autolyze at 45°C for 8 hours. The autolysate was extracted with N/10 H Cl, and the acid solution filtered through a Berkefeld candle; on neutralization (to pH 8.0) this filtrate gave an abundant--inert--precipitate which was readily removed by centrifugalization. The clear supernatant was precipitated with 3 per cent trichloracetic acid and left in contact with this reagent for 3 hours. When the precipitate was taken up in distilled water, most of it remained insoluble, but the solution was highly active. This solution, separated from the precipitate, was again precipitated with 3 per cent trichloracetic acid, yielding a product entirely and readily soluble in water. This material, apparently a protein, is soluble in acetone from which it precipitates on neutralization. It was necessary to carry out this last step in the cold in order to avoid denaturization with ac-

companying loss of solubility and activity.

When tested on heat-killed pneumococci, this solution was found to change the cells from gram-positive to gram-negative, without, however, bringing about a complete dissolution of the formed elements. The enzyme has an optimum activity at slightly alkaline pH, but is also effective at pH 7.0. Although it resembles trypsin in some of its properties, it differs from the latter in many respects; trypsin, for instance, causes marked clearing of suspensions of heat-killed pneumococci without changing the cells to gram-negative. Under proper conditions, 1 mg. of the enzyme extracted from leucocytes will render gram-negative 200 mg. of heat-killed pneumococci (Type I).

In 1921, Flemming demonstrated the presence in eggwhite, tears, and various animal tissues, of a principle—lysozyme—capable of lysing cultures of many saprophytic microorganisms. Two years ago, K. Meyer developed a technique for the purification of lysozyme and demonstrated the enzymatic nature of its action. Through his courtesy, we have been able to test the action of pure lysozyme on pneumococci and found that, also in this case, there occurs a loss of the Gram reaction, without any actual dissolution of the cells. Lysozyme differs from the leucocyte enzyme previously described in many of its properties; for instance, it is much less stable at alkaline reactions, it is active against pneumococci only in high concentrations, and has an optimum activity between pH 5.5 and 6.5. Furthermore, lysozyme acts upon a great many different groups of hemolytic streptococci, whereas the leucocyte enzyme is ineffective against all the strains of streptococci tested.

We have, therefore, two enzymes, namely, the one extracted from polymorphonuclear leucocytes, and lysozyme, both of which have the property

of rendering heat-killed pneumococci gram-negative, without, however, causing a complete dissolution of the cell bodies. If now rabbits are immunized by the intravenous route with vaccines of virulent pneumococci treated by these enzymes, the animals fail to produce the capsular polysaccharide precipitins which regularly appear in the serum following intravenous immunization with the untreated vaccine. It is, therefore, apparent that the action of these two enzymes renders ineffective the capsular antigen in rabbits.

2. Avery and Cullen have demonstrated the existence in Pneumococcus of an enzyme-complex capable of lysing suspensions of heat-killed pneumococci. A further study has been made of the methods of preparation and of the properties of this enzyme. It was found, for instance, that each pneumococcus cell contains enough enzyme to render gram-negative 50 to 100 cocci killed at 60°C; this point is of importance in view of the rapidity with which pneumococci autolyze under certain conditions.

As in the case of the leucocyte enzymes, it was found that heat-killed or formalized vaccines entirely lost their activity as "capsular antigen" in the rabbit after treatment with an enzyme preparation derived from a pneumococcus autolysate. This action could be obtained using as the source of enzyme either an R or S variant, irrespective of type. Furthermore, it was not necessary to use an amount of enzyme sufficient to cause complete dissolution of the cells. The mere change in the cocci from gram-positive to gram-negative was sufficient to deprive the cells of their specific antigenic behavior.

It is, therefore, obvious that, in any attempt to extract in an active form the capsular antigen of Pneumococcus, great care must be taken to prevent any autolysis in the course of the process. Two techniques have generally been advocated for obtaining solutions of pneumococci: (a) solution in bile or bile salts, (b) disintegration of the cells by freezing

and thawing. The effect of bile is generally attributed to its activity as a depressor of surface tension or as a peptising agent; repeated freezing and thawing is supposed to rupture the cells by the formation of ice crystals. These two techniques were subjected to an exacting experimental analysis, and it was found impossible to obtain rupture of the cells by either one of them under conditions that did not allow enzymatic action. The experimental results may be summarized as follows: (1) bile solubility, and susceptibility to freezing and thawing are not dependent upon viability of the cells; both properties are still present when the cells have been killed by agents which do not inactivate the cellular enzymes; (2) both properties disappear when the enzymes have been inactivated by heat or by poisons; (3) both processes are ineffective when carried out in acid or alkaline reactions, outside the range of activity of the autolytic enzyme; (4) they are ineffective when the temperature is at no time allowed to reach a level at which enzyme action can take place.

Many other techniques have been developed to put pneumococci in solution, but all of them involve as a necessary step an action of that enzyme which renders the cells gram-negative. And it is this change which seems to render the capsular polysaccharide antigen ineffective in the rabbit.

Three enzymes have, therefore, been demonstrated to have the power of inactivating the polysaccharide antigen of virulent pneumococci, namely, the leucocyte enzyme, lysozyme, and the pneumococcus autolytic enzyme. The nature of these enzymes is still unknown, although K. Meyer has presented some evidence indicating that lysozyme acts as a mucinase. Of much significance is the fact that any action rendering pneumococci gram-negative also impairs their ability to act as a complete antigen. Careful microscopic

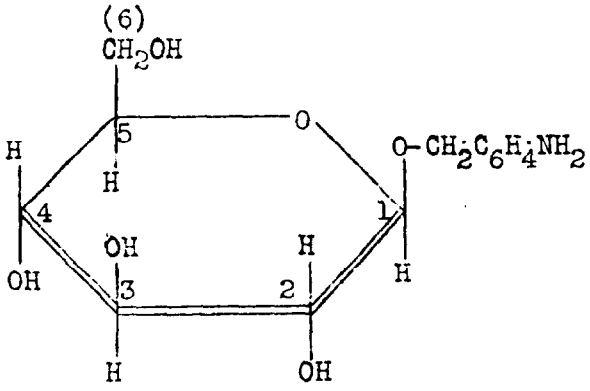
examination has shown that heating a cell suspension of pneumococci to 60°, which is the usual method of preparing vaccines, does not prevent a certain amount of autolysis from taking place. Different techniques for preparing bacterial vaccines and the antigenic efficacy of the resulting preparations are now being studied. It is hoped that these investigations will assist in demonstrating the nature of the "capsular polysaccharide antigen."

The synthesis of uronic acids and their chemo-immunological relationship to the specificity of bacterial polysaccharides (Goebel and Hotchkiss). The immunological specificity of encapsulated microorganisms may be attributed to the specific polysaccharides which constitute a part of the encapsulating material. It has been observed in our laboratory that all of the specific bacterial polysaccharides thus far investigated contain certain sugar acids, uronic acids, which form an integral part of the specific carbohydrate molecules. These uronic acids are believed to have a more important function in determining the type specificity of bacterial polysaccharides than do the hexose constituents with which the uronic acids are combined.

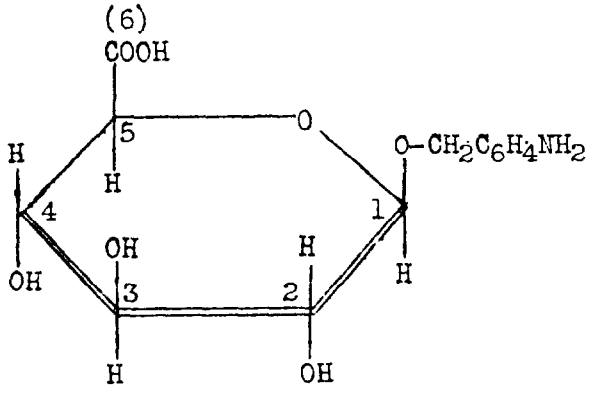
In support of this view, the immunological properties of an artificial antigen, prepared by combining the p-aminobenzyl glycoside of glucuronic acid with foreign protein, have been investigated and compared with those of a similar antigen containing the p-aminobenzyl glycoside of glucose. It has been found that rabbits immunized with the artificial glucuronic acid antigen give rise to specific antibodies which bear no serological relationship to the antibodies elicited by the glucose antigen.

In previous reports it has been shown that in artificial carbohydrate-protein-antigens containing hexoses and disaccharides, certain identities in the stereochemical configuration of the carbohydrate radicals are reflected

in the antibodies to which the antigens give rise. The stereochemical relationship of the asymmetric carbon atoms of the p-aminobenzyl glycosides of glucose and of glucuronic acid may be seen from the following structural formulae:



p-aminobenzyl glycoside of glucose



p-aminobenzyl glycoside of glucuronic acid

It is apparent that the stereochemical configuration of the two glycosides is in each instance identical. They differ only in that the glycoside of glucose contains a non-polar primary alcohol (CH_2OH) in the sixth position, whereas in the glucuronic acid glycoside molecule, a highly polar carboxyl (COOH) occupies this position. The specificity of the antibodies induced by antigens containing these two glycosides is so sharply defined that they exhibit no serological crossing whatsoever. This singular selectivity must be attributed, therefore, to differences in the chemical groupings occupying the sixth position in each glycoside molecule.

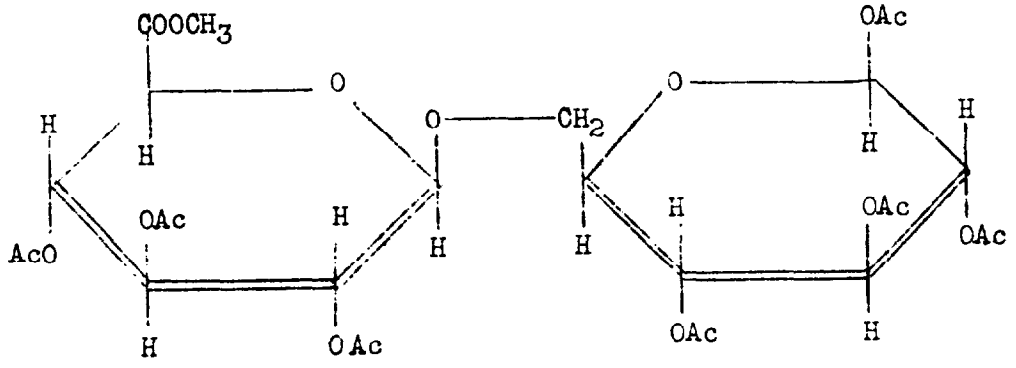
It has previously been found that the specific polysaccharides of Types II, III, and VIII pneumococcus are constituted of molecules of glucose and glucuronic acid in varying proportions. The important role played by the highly polar uronic acids as determinants of the immunological properties of encapsulated microorganisms is clearly demonstrated by the following fact: the artificial glucuronic acid-protein antigen reacts in Types II, III, and VIII antipneumococcus horse sera, in dilutions of one part in one million, whereas the corresponding glucose antigen is serologically inert. In

these instances, the serological activity of the artificial glucuronic acid-protein antigen must be attributed to the interaction of antibodies elicited by the highly polar uronic acid constituent of the bacterial polysaccharides, with similar groups in the artificial antigen. Despite the fact that the artificial glucuronic acid-protein antigen possesses these unusual immunological properties, it does not evoke antibodies capable of protecting animals against experimental pneumococcus infection. The reason for this may lie in the fact that the glucuronic acid constituent of the artificial antigen does not approximate closely enough the uronic acid constituents of the specific bacterial polysaccharides. The latter substances contain aldobionic acids, disaccharides of which glucuronic acid is but one component.

The preparation of artificial aldobionic acid-protein antigens necessitates the acquisition of considerable quantities of these acids, either by isolation from natural sources or by chemical synthesis. The aldobionic acid obtained from gum acacia, galactose-6-glucuronide, has been selected as more suitable for preliminary experiments than the rarer aldobionic acids of the bacterial polysaccharides. A method has now been developed for the preparation of the bromo-heptacetyl methyl ester of the aldobionic acid from gum acacia. This derivative will serve as an important intermediate for the preparation of the corresponding aldobionic acid-protein antigen.

Chemical synthesis as a method of obtaining aldobionic acids has awaited the development of the chemistry of glucuronic acid. The recent preparation of acetohalogen derivatives of glucuronic acid in this laboratory has now made possible the synthesis, not only of conjugated glucuronides, but of aldobionic acids as well.

The synthesis of the β-heptacetyl methyl ester of the aldobionic acid, glucose-6-β-glucuronide:



has been accomplished by condensing 1, 2, 3, 4-tetracetylglucose with 1-bromotriacetyl-glucuronic acid methyl ester. The β-heptacetate so obtained has been converted into the α-modification, which is isomeric but not identical with the α-heptacetyl methyl ester of the aldobionic acid derived from types III and VIII pneumococcus polysaccharides. The application of analogous synthetic procedures should soon make possible the preparation in the laboratory of aldobionic acids identical with those present in the soluble specific substance elaborated by Pneumococcus.

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